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Systemic distribution and elimination of plain and with Cy3.5 functionalized poly (vinyl alcohol) coated superparamagnetic maghemite nanoparticles after intraarticular injection in sheep in vivo

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Systemic distribution and elimination of plain and with Cy3.5 functionalized poly(vinyl alcohol) coated superparamagnetic maghemite nanoparticles after intraarticular injection in sheep *in vivo*

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Abstract

PVA coated and fluorescent dye (Cy3.5) functionalized vinyl alcohol/vinyl amine copolymer coated superparamagnetic iron oxide nanoparticles (SPION) were evaluated for systemic distribution and elimination after intraarticular injection in sheep. Observation was done at 3, 24, 72 and 120 hours after injection using light microscopy, fluorescent microscopy and confocal microscopy. No pathologic influence of SPION on the tissue harvested could be seen. A significantly increased iron content could be identified in the kidneys, lymph nodes and spleen after injection of SPION. No particles were detected in the liver, the urinary and the gall bladder. No positive fluorescent signal could be attributed to SPION throughout the organs. Our results indicated that the iron component of the SPION is possible to be incorporated into the physiologic iron metabolism after reabsorption in the proximal tubule system of the kidney and that concentration levels of Cy3.5 are too low to be detected throughout the body.

Introduction

Nanoparticles have recently received much attention due to their characteristics that enable them to serve as drug carriers for targeted drug delivery [1, 2]. Several types and sizes of particles can be used to deliver pharmaceutical products, plasmids or peptides locally to specific organs. Among those particles, the superparamagnetic iron oxide particles (SPION) are especially attractive because of their potential to be locally concentrated and to be held in place by external magnets, enhancing the uptake of the delivered molecule at the site of interest.

In modern clinical medicine, SPION are used for diagnostic purposes in MRI and in experimental cancer therapy [3-7]. In recent studies these types of particles were used to diagnose macrophage activity within the joint capsule in experimental rats [8]. The application of SPION as magnetic drug carriers into diarthrodial joints is also limited to experimental studies. Since the magnetic properties of these particles make them an attractive tool to treat joint inflammations and/or modulate articular cartilage degradation, our group investigated whether SPION coated with poly(vinyl alcohol) (PVA) could be used for intraarticular application in the carpometaphalangeal and in stifle joints of experimental sheep [9]. In this study, PVA-SPION of a 6-10 nm core and overall diameter of 30-40 nm proved to be biocompatible. They were successfully taken up by synoviocytes of the synovial membrane and were distributed within the connective tissue of the joint capsule. In the course of the five day evaluation period PVA-SPION showed a peak concentration in the synovial membrane at 120 hours after intraarticular application.

It is generally accepted that after systemic administration, SPION are eliminated from the body through the reticuloendothelial system [10]. Nanoparticles were mainly detected in the liver and the spleen [11]. It was hypothesized that nanoparticles were degraded and that iron was further incorporated into hemoglobin and thus was introduced into the physiologic metabolism of Fe^{3+}/Fe^{2+} ions of the body. However, the systemic elimination of SPION from the joint space has not been previously reported.

Therefore, the goal of our study was to investigate the systemic distribution and elimination of plain PVA-SPION and of Cy3.5 functionalized vinyl alcohol/vinyl amine copolymer-SPION after intraarticular application in sheep. Our hypothesis was based on the assumption that particles would be phagocytosed by

macrophage-like cells in the joint tissue, subsequently be transported via lymph nodes and the lymphatic system and finally after introduction into systemic circulation distributed to the spleen and the liver.

This evaluation was part of our earlier study, where the biocompatibility and intracellular uptake of the same particles has been tested in the joint tissue [9]. Tissue samples of the sheep have been harvested at the time of sacrifice and served as basis for this investigation.

Materials and methods

SPION: Plain PVA-coated SPION and amino-PVA-coated with Cy3.5 functionalized SPION were used for the study.

Cy3.5 labeled vinyl alcohol/vinyl amine copolymer coated SPION and poly(vinyl alcohol) coated SPION were investigated in this study. The compounds will be referred to as amino-PVA-Cy3.5-SPION and PVA-SPION, respectively.

Preparation of nanoparticles: The preparation of superparamagnetic iron oxide nanoparticles and the vinyl amine /vinyl alcohol co-polymer followed a standard protocol and is described elsewhere [12, 13] . Briefly, iron oxide polymer coated nanoparticles were prepared by alkaline co-precipitation of ferric and ferrous chlorides in aqueous solution. Solutions of $FeCl_3 \cdot 6H_2O$ (0.086 M) and $FeCl_2 \cdot 4H_2O$ (0.043 M) were mixed and precipitated with concentrated ammonia while stirring vigorously. The black precipitate was washed several times with ultra-pure water until the pH decreased from 10 to 7. The solid was collected and refluxed in a mixture of 0.8 M nitric acid and 0.21 M aqueous $Fe(NO_3)_3 \cdot 9H_2O$ for 1 h. The system was allowed to cool to room temperature, the remaining liquid was discarded, and 100 ml of ultra-pure water was added to the slurry, which immediately dispersed. The brown suspension was dialysed for 2 days against 0.01 M nitric acid, and stored at 4 °C. In a final step the obtained product was mixed with the different polymer solutions to obtain either SPION coated with polyvinyl alcohol (Mowiol® 3-83), or SPION coated with vinyl alcohol/vinyl amine copolymer (M12, Erkol S.A.). Here, vinyl amine/vinyl alcohol co-polymer coated SPIONs were diluted in 25 mM carbonate buffer (pH=9.3) to a final concentration of 2.5 mg Iron/ml dispersion. The used Cy3.5 dye (Amersham Biosciences) is a monofunctional NHS-Ester, and was provided in a dried, premeasured form ready for the labeling of compounds containing free amino groups. The dispersion of polymer coated SPIONs

was added to the dye vial and thoroughly mixed. The particles were incubated for 1 hour and the labeled particles were separated from excess, unconjugated dye using disposable PD-10 desalting columns (Amersham Biosciences). The column was pre-equilibrated with 25 mM phosphate buffer, which was also used to elute the particles. The concentration of the dye per particle was determined by photometrical quantification of the iron and the dye using a molar extinction coefficient of 150'000 $M^{-1}cm^{-1}$ at 581nm for the Cy3.5.

Experimental animals: Fourteen (14) Swiss Alpine sheep 2 - 4 years of age were selected for the study. The general health of sheep was determined and the sheep were routine dewormed and vaccinated prior to the study. During a 2 weeks adaptation period before the start and until the end of the project, the animals were kept in groups in box stalls. They were fed grass hay and had free access to water, except 24 hours prior to anesthesia when all food was removed.

The sheep were divided into four groups, A to D. Each group consisted of four animals, of which two animals received plain PVA-SPION and the other two animals received amino-PVA-Cy3.5-SPION into their joints. Group A consisted of two animals which received plain PVA-SPION and were sacrificed 3 hours after surgery. Sheep of group B, C and D were sacrificed after 24, 72 and 120 hours, respectively after SPION application. Six additional sheep, slaughtered for reasons other than infectious or systemic diseases served as control animals.

Joint injections: To perform the joint injections, the sheep were placed under general anesthesia. The animals were sedated with medetomidine (5 μg/kg bwt i.m., Domitor®, Orion-Farmos, Turku, Finland) before induction using diazepam (0.01 mg/kg bwt i.v., Valium, Roche, Basel, Switzerland) and ketamine (20 mg/kg bwt i.v., Narketan®, Chassot AG, Bern, Switzerland). Anesthesia was maintained with isoflurane (Forene®, Abbott AG Baar, Switzerland) in 100% oxygen. Analgesia was accomplished by intravenous injections of buprenophine (0.03 ml/kg bwt. Temgesic®, ESSEX Chemie AG, Luzern, Switzerland) perioperatively and postoperatively every 4 hours as needed.

The skin at the area of the joints to be injected was clipped and prepared in a routine surgical manner. Proper placement of the needle in the joint cavity was confirmed by aspiration of synovial fluid. 1.0 ml of the nanoparticle suspension was injected into each carpometaphalangeal joint and 2.0 ml into the lateral pouch of the femorotibial joint.

Evaluation: Macroscopic evaluation was done at the time of sacrifice, where liver, spleen, kidneys, urinary bladder, gall bladder, inguinal and axillary lymph nodes were collected from all animals apart from the joint tissues. Results of the joint tissues were reported elsewhere [9]. Tissue samples of these organs were taken and processed for standard paraffin histology. Slides of the paraffin blocks were stained with Haematoxylin-Eosin (H.E.), Pearl's Prussian blue (Fe^{3+}) and Turnbull (Fe^{2+}).

Histological evaluation for pathologic changes and presence of nanoparticles in the tissue samples was performed using light microscopy (DMR, Leica, Glattbrugg, Switzerland) and digital imaging (DC 200, Leica). Fluorescence was evaluated on native slides with the same microscope, equipped with an I3 filter with excitation range of 450-490nm.

Confocal microscopy was performed for better evaluation of SPION distribution using CLSM (confocal laser scanning microscopy, Leica TCS SP2 AOBS, Leica Microsystems, Mannheim, Germany). The IMARIS software (Bitplane AG, Zurich, Switzerland) was used for 2-D multi-channel image processing. Background fluorescence of cells was determined by analyzing untreated tissues.

H.E. stained slides were used for assessment of morphologic changes in tissue samples. Detection of nanoparticles was done using Fe^{2+} and Fe^{3+} stained slides. A scoring system was developed to assess nanoparticle concentration ranging from 0 - 3, with score 0 = no nanoparticles, score 1= nanoparticles observed in up to 33% of the field of view, score 2= nanoparticles observed in 34 – 66% of the field of view, and 3= nanoparticles observed in 67-100% of the field of view. On each slide, six power fields on a 20-fold magnification were judged so a total score of maximally 18 per slide was possible. Fluorescence was compared between groups, but also within groups to assess whether it was similar to particle distribution (Fe^{3+}, Fe^{2+}).

Statistics: One factorial variance analysis for the incubation time was calculated for each organ in which SPION, not divided into PVA-SPION or amino-PVA-Cy3.5-SPION, were found at 0 hours, reflecting control animals, 3 hours, 24 hours, 72 hours and 120 hours. Independence between the different results at each time could be assumed since data of different animals were available at each time evaluated. The Bonferroni/Dunn procedure was used to analyse variances at different times. P-values of 0.05 or less were considered significant.

Results

Identical distribution of the PVA-SPION and amino-PVA-Cy3.5-SPION was found within groups irrespective of the Fe^{3+}/Fe^{2+} special stains used. Therefore, only scores of particle distribution will be discussed in the following.

Kidney: None of the slides showed any signs of histopathologic changes as viewed with the H.E. stained specimens. Tissue samples of 2 out of 6 control and 9 of the 14 injected animals showed positive reaction after P.B. (Fe^{3+}) staining. Exclusively affected were the epithelial cells of the proximal tubules *(Fig.1a)*. The cytoplasm was colored light blue, with or without dark blue granules intracellularly.

Semiquantitative evaluation at 3, 24, 72 and 120 hours after joint injection, indicated a significant increase in Fe^{3+} ions in animals that had received SPION. ANOVA calculations of the observations made in the kidneys resulted in *F*- values of 13.29 with a significance level of less than 0.0001, indicating that there was a significant increase in Fe^{3+} ions in the experimental animals when compared to the control animals at all time points of the study. No other significant differences were shown with Bonferroni/Dunn calculations. This observation indicated a quick increase of Fe^{3+} ions as early as 3 hours post-injection after which the concentration stayed on the same level throughout the experimental period. (*Fig. 1b*)

In Turnbull stained tissue samples Fe^{2+} ions were detected in epithelial cells of the proximal tubules only in two animals, one in the 24-hours group and one in the 72-hours group *(Fig. 1c)*. This resulted in a mean value of 0.021 for the 24-hours group and 0.125 for the 72-hours group in the 0-3 scoring system. Bonferroni/Dunn calculations demonstrated a significant elevation of the 72-hours group compared to all other groups in the study (P= 0.0024) *(Fig. 1d)*.

Lymph nodes: Both inguinal and axillary lymph nodes were harvested from all animals in this study. Histological changes seen in most of the tissue samples of both control and injected animals included reactive lymphoid or hyperplasia with plasma cells, a mildly increased number of macrophages (sinus histiocytosis), neutrophilic and eosinophilic granulocytes as well as some "Russell bodies". No differences were noted between injected and control animals and the described changes were not interpreted to be significant in any of the sheep.

After P.B. staining tissue samples of 2 out of 6 control animals, and of all injected sheep showed bluish stained macrophages in at least one lymph node. Although blue stained macrophages were found in injected animals as well as in control sheep, the quantity of stained cells increased significantly as from 3 hours after SPION joint injections and remained elevated until 120 hours post injection. ANOVA calculation was done for both left and right axillary and inguinal lymph nodes combined, which resulted in an F-value of 19.68. This corresponds to a significance level of less than 0.0001. A differentiated examination demonstrated significant elevations of counts when compared to control animals 3, 24, 72 and 120 hours after injection. The highest level of counts was reached at 72 hours post injection (increase 3 hours/72 hours p= 0.0024; increase 24 hours/ 72 hours p= 0.0003). No other significant change was observed during the test period. (*Fig. 2*)

In the Turnbull stained samples, Fe^{2+} ions in macrophages of both axillary lymph nodes in one sheep of the 24-hours group and both inguinal lymph nodes in one sheep of the 120-hours group were found. ANOVA calculations resulted in mean values of 0.177 for the 24-hours group and 0.100 for the 120-hours group on our 0-3 scoring system. This demonstrates a significant elevation of Fe^{2+} ions 24 hours post injection compared to the control animal, the 3-hours and the 72-hours groups when differentiated after Bonferroni/Dunn analysis. (*Fig. 3*)

Spleen: With the exception of one control sheep, spleen samples of all animals in this study were examined histologically. Several of the H.E. stained specimen demonstrated reactive hyperplasia with an increased number of macrophages and plasma cells. None of the changes we saw on H.E. stained slides were interpreted as outside the normal variations in sheep of this age group.

Blue colored macrophages were seen in P.B. stained samples of all animals. The white splenic pulp was always free of positively stained cells. The red pulp contained large amounts of blue stained macrophages, to a

degree where the spleen was diagnosed with moderate hemosiderosis in 11 out of 14 injected animals (*Fig. 4a)*. ANOVA calculation for the spleen showed a P- value of 36.20 with a significance level of less than 0.0001. A large amount of stained Fe^{3+} molecules were found in control animals, but the Bonferroni/Dunn calculations demonstrated significant elevations for injected animals with P-values of less than 0.0001 at all times measured. Statistical evaluation showed a continuous increase of values with the time. The differences between 24 hours and 120 hours ($p< 0.0001$) and between 72 hours and 120 hours ($p= 0.0017$) were significant. Comparable interpretation of the results from 3 hours post injection sheep required caution since results of only 2 animals of this group were available. (*Fig. 4b*)

In the Turnbull stained specimen, macrophages in 3 of the 6 control animals and 12 out of 14 injected animals were stained positive (*Fig. 4c)*. The mean scores for Fe^{2+} ions were all lower than for the Fe^{3+} ions detected with the P.B. staining. ANOVA calculations for Fe^{2+} ions on spleen samples demonstrated a significant increase of stained particles between animals in the 24-hours and 72-hours groups when compared to control animals. The counts on slides of the 120-hours group were significantly higher than in all other groups in this study. (*Fig. 4d*)

Liver: Liver samples of all animals in this study were examined samples of three sheep visualized with H.E. stained slides showed mildly to moderately increased numbers of inflammatory cells (lymphocytes, plasmacells, macrophages, eosinophils) periportally indicating chronic parasitism confirmed at the time of sacrifice (fluke infestation). No alterations were noticed distinguishing treated from non-treated animals.

After P.B. staining, no bluish stained cells were seen on specimen of either control or 3-hours group animals. Blue stained cells were seen in one of the sheep in the 24-hour group and a significantly increasing amount of blue stained cells with time during the course of the experiment. The affected cells were mainly Kupffer cells, but also periportal macrophages and rarely hepatocytes contained Fe^{3+} ions *(Fig. 5a)*. Statistical calculation gave *F*- value for the liver as 29.49 and the *P*- value was <0.0001. Significant increases ($p< 0.0001$) were shown between the times 0h/72h, 0h/120h, 3h/120h, 24h/120h and 72h/120h. These results combined together show a constant increase of Fe^{3+} ions from 3 hours post- injection until the end of the test period (*Fig. 5b*).

In the Turnbull stained samples, we also found stained Kupffer cells, macrophages and some hepatocytes. Fe^{2+} ions were seen in animals of all groups and in higher mean scores than the Fe^{2+} ions were seen in animals of

all groups and with a higher mean scores than the Fe^{3+} ions. Scores for the control animals and for the 24-hours group were significantly lower than for the 3-hours, the 72-hours and the 120-hours groups (*Fig. 5c*).

Urinary and gall bladder: Tissue samples of the gall bladder and the urinary bladder wall of all animals in the experiment were available for histologic examination. None of these two organs showed histopathologic changes on the H.E. stained specimen and no Fe^{3+} or Fe^{2+} ions were found using either P.B. or Turnbull staining, respectively.

Fluorescence: In all organs and in all experimental animals evaluated, strong background fluorescence of the tissue was detected. Some fluorescence signal of amino-PVA-Cy3.5-SPION was detected; however, this could not be clearly and distinctively be attributed to either SPION or background. Therefore, no further histological nor statistical evaluation was performed.

Discussion

In this *in vivo* study, two different intraarticularly injected SPION were assessed for their physiological metabolism and potentially harming effects in different organs in sheep during a time period between 3 to 120 hours after injection. Systemic distribution of particles *in vivo* could be followed for both, the plain PVA-SPION as well as the amino-PVA-Cy3.5-SPION. After intraarticular injection into the carpometaphalangeal and stifle joints of the sheep, the iron component of SPION was distributed via the regional axillary and inguinal lymph nodes, respectively, the reticuloendothelial system. Based on iron stainings our results suggest that filtration through the glomerulonephritic system and reabsorption within the proximal tubules of the kidney. Thereafter, iron ions were taken up into the reticuloendothelial system ending up mainly in the spleen but also the liver.

A clear and consistent statistically significant distribution of nanoparticles in different organs and over time could not always be established. This could be due to several reasons, among which the small number of animals per group could be the most important. Uneven particle distribution within the same organs was noticed and certainly this contributed to this phenomenon, although we tried to distribute the chances evenly by evaluation of 6 fields of view per slide for each animal and by evaluation of different tissue sections per

organ. Additionally, functionalization of PVA-SPION with Cy3.5 dye may have altered the rate of elimination when compared to the plain PVA-SPION.

An experimental animal model with sheep was established to evaluate the future clinical use of magnetic targeted drugs. For costs and ethical reasons only a limited number of experimental animals was used. Therefore our goal was not to provide toxicological and safety data for the SPION used, but to proof the principle for magnetic drug targeting with SPION and to give a first idea about body distribution, possible toxicities and elimination pathways.

The sheep were injected with 1 ml of the nanoparticle suspension into each carpometaphalangeal joint and 2 ml into each stifle joint, corresponding to 6 ml nanoparticle suspension or 27.6 mg elemental iron per animal. The body weights of the sheep in this study ranged between 45 kg and 81 kg, resulting in a dosage of 0.61 to 0.34 mg elementary iron per kg body weight.

Comparing the dosage used in this trial with clinically employed treatment regimens used in veterinary practice, such as the recommended dose for prevention of iron deficiency anemia in baby pigs (1-3 days of age) is 100- 150 mg of elemental iron IM per pig (Label directions: Ferrextran-100®, Fort Dodge). Assuming that SPION will be spread throughout the body system on hematologic or lymphatic pathways we did not expect that SPION will be detected in the blood as standard blood tests for iron are not sensitive enough to detect such small amounts of iron in sheep. Blood samples were taken, but there was no deviation in the iron parameter in experimental animals from control animals.

H.E. stained tissue samples were used for examination of morphology and histopathologic changes in different organs. None of the organs showed any evidence of harmful effects of the injected nanoparticle suspension on their systemic elimination pathway during the examined time period of 120 hours (5 days). It can be argued that this follow-up period is relatively short to assess systemic toxicity or harming effects. However, since toxicity of ferrous solutions usually is an acute incidence if accidentally injected intravenously, five days seemed an adequate time for this type of study. If concentrations at the local injections sites were toxic, this would have been visible during this follow-up time period. This lack of toxicity was consistent with earlier results from our group after intraarticular and periarticular application [9]. Therefore it can be safely assumed

for future toxicological studies on SPION that significant toxic reactions to the iron component of PVA-SPION or Cy3.5-amino-PVA-SPION are not likely.

Pearl´s Prussian Blue (P.B.) and Turnbulls reaction are used to show different forms of iron in tissues [14]. P.B. stains iron in the ferric (Fe^{3+}) form and Turnbulls reaction stains iron in the ferrous (Fe^{2+}) form. The injected nanoparticles contain ferric and ferrous iron and an earlier study showed that the P.B. and Turnbulls reactions are well suited methods for detecting these particles in synoviocytes in monolayer cell cultures [15]. Hemoglobin and myoglobin contain iron in the Fe^{2+} form, but cannot be stained by the Turnbull reaction. In plasma, ceruloplasmin oxidizes ferrous iron immediately to ferric iron (Fe^{3+}). Therefore, ferrous iron is usually found only to a small amount in the body. Iron is stored in the body in various tissues as either a soluble, mobile fraction (ferritin) or as insoluble aggregated deposits (hemosiderin) [16]. Both these forms contain iron in the ferric form and hemosiderin is readily stained with the P.B. reaction. Spleen and liver usually have the highest storage iron concentrations and the iron found on the P.B. stained specimen is likely storage iron in form of hemosiderin.

Although, iron is present physiologically in different forms and can be found throughout the body, differences existed in the sheep after PVA-SPION application. The significant increase in Fe^{3+} particles found in lymph nodes, spleen, liver and kidneys of test animals when compared to controls seems to result that the iron injected into the joints of the test animals was incorporated into their physiological iron metabolisms as hypothesized. Tracing of the PVA-SPION itself was not possible as none of the components except the iron was detectable in vivo.

The spleen appears to be the organ that had the highest iron storage capacity in sheep. In our scoring system the mean score for the spleen was higher than that of the other examined organs and reached the maximum score at 120 hours after injection of test animals in both, the Fe^{3+} and Fe^{2+} ions.

Fe^{3+} ions were not detected in either the liver of control animals or of sheep sacrificed 3 hours after injection, however, there was significant increase between 3 and 120 hours post injection indicating a function of the sheep liver in iron storage as well. This is not surprising given the physiological role of the liver and the spleen. At 3 hours after injection there was also a significant increase of Fe^{2+} ions in the liver. Histologically this is to be interpreted as an increase of non-haem iron in the liver. As reference values are missing for the

sheep it can not be stated if the increase is due to the SPION injection, physiological or shows evidence for hemochromatosis, but will be proven with MRT in future studies.

The kidneys are known to have iron storage capacity in other species, which we could confirm for sheep in the control group. The immediate increase in Fe^{3+} concentration after intraarticular PVA-SPION injections, however, implies either increased resorption and/or storage of iron in the proximal tubules, but glomerular filtration and reabsorption of ferritin (~20,000 Da) in the proximal tubules could be possible too. Therefore it can be assumed that SPION are not simply excreted via the kidneys into the urine but dissociation of SPION into single components is possible and then iron is entered into the physiologic iron metabolism. The small particle size may even allow resorption of the SPION alone or bound to a transport protein, such as albumin. Transmission electron microscopy of samples will hopefully clarify these questions in the future (*study ongoing, senior author*).

The lymph nodes are not an iron storing tissue, but the macrophages as part of the MPS are able to phagocytose iron containing particles. The small number of particles seen on lymph node specimen of control animals result likely from hematomas and the significant increase in particle concentration at each time after injection suggests phagocytosis of PVA-SPION.

Iron in the ferrous form was found in spleen and liver samples of control animals and in spleen, liver, lymph node and kidney samples of test animals. The locations of detected Fe^{2+} ions were the same as for Fe^{3+} ions, but the quantity was less. Scores similar to those in the Fe^{3+} evaluation were reached in spleen and liver samples only. A clear pattern of particle distribution over the different time frames could not be detected given the small sample size per group. However, this observation may lead to speculations that an immediate transformation of ferrous iron to ferric iron by enzyme (ceruloplasmin) activity in synovial tissues may have occurred before release into lymph or blood circulation. Liver and spleen are known to be important sites of iron metabolism, therefore detection of iron particles in different forms (Fe^{3+}/Fe^{2+}) were to be expected.

It could not be proven whether the Cy3.5 dye kept its covalent binding to the amino-PVA-SPION. Cy3.5 has been used to indicate if the binding to the SPION is stable throughout the distribution and elimination process. Clear attribution of fluorescent signals to SPION during fluorescence microscopy was not possible. It can be assumed that the very low concentration of dye/particle (<10) together with an iron concentration of 0.61 to

0.34 mg elementary iron per kg body weight allowed very high distribution and dilution of Cy3.5 throughout the body. Within the synovial membrane positive signals of SPION could be proven at any times of evaluation [9]. Whether Cy3.5 left the synovial membrane at all could not be proven in this study. In this study Cy3.5 has been used to indicate how pharmaceutical products may react if bound to SPION and applied for localized treatment of chronic or acute inflammatory joint diseases. We hypothesize that therapeutically interesting dye concentrations are only present in the synovial membrane supporting our goal to accumulate the dye in the area of interest only. At this stage it can not be assumed that pharmaceutical products, administered at the same conditions as Cy3.5, maintain a therapeutic level of activity when removed systemically from the synovial membrane.

Conclusion:

We demonstrated that the distribution and elimination of plain and with Cy3.5 functionalized PVA-SPION could be followed *in vivo* after intraarticular application in sheep. Administration of these nanoparticles was safe and the iron component seemed after initial filtration and reabsorption within the proximal tubules of the kidney to be incorporated into the physiological iron metabolism, followed by its degradation in the reticuloendothelial system. Side effects were absent in all organs tested and therefore, the intraarticular use of PVA-SPION is promising as an attractive carriers for future targeted drug delivery in joint disease. Future studies with these particles will focus on medical applications in combination with drugs or genetic material and on the systemic elimination of the PVA coating from the body.

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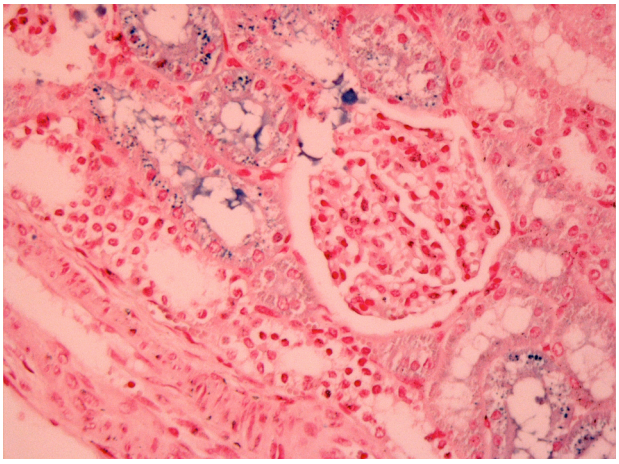
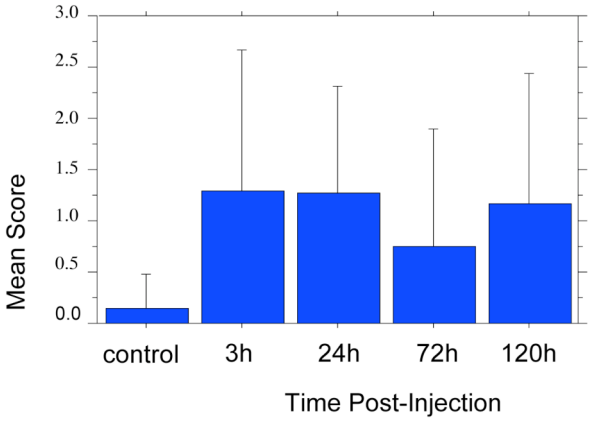
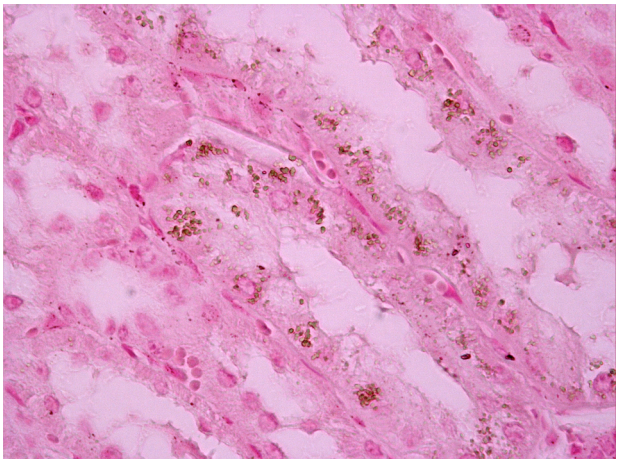
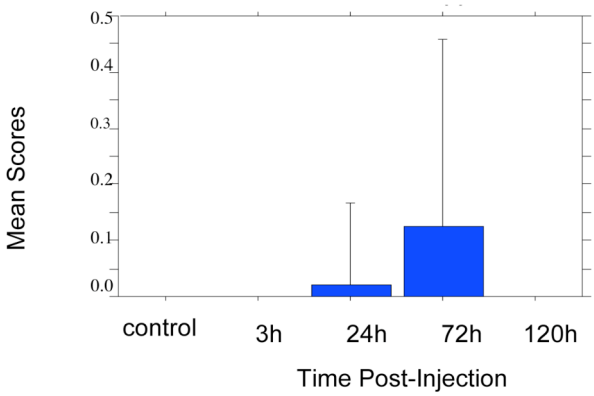




a **b**





c **d**

Figure 1. Kidney tissue; *a*, blue stained iron (Fe^{3+}) in epithelial cells of proximal tubules 24 hours post PVA-SPION injection (P.B., 40x); *b*, distribution of detected iron ions in P.B. stained kidney tissue of controls and of animals injected with SPION; *c*, brown stained iron (Fe^{2+}) in epithelial cells of proximal tubules 72 hours post PVA-SPION injection (Turnbull, 40x); *d*, distribution of detected iron ions in Turnbull stained kidney tissue of controls and of animals injected with SPION.

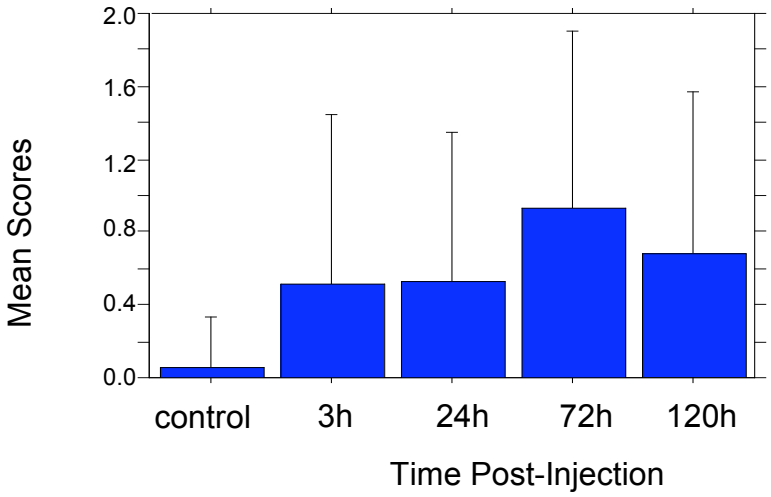


Figure 2. Distribution of detected iron ions (Fe^{3+}) in P.B. stained lymph node tissue of controls and of animals injected with SPION.

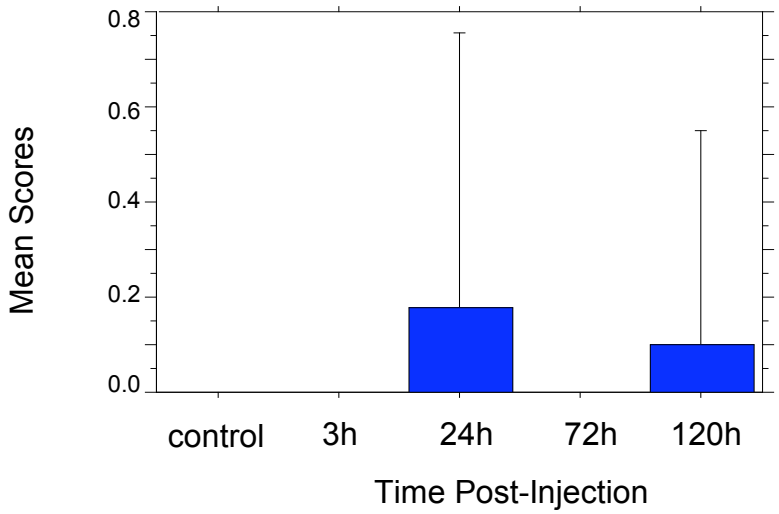
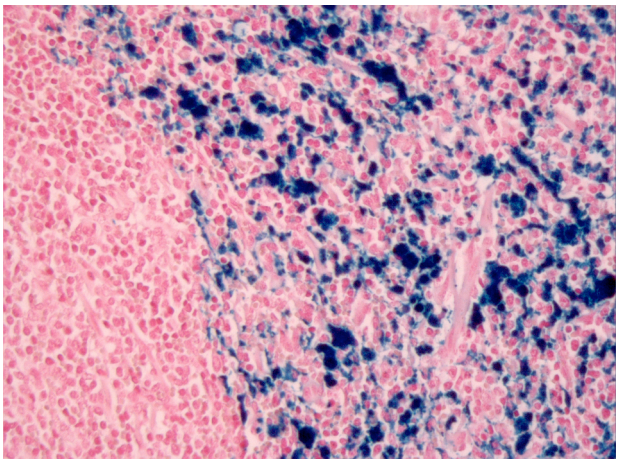
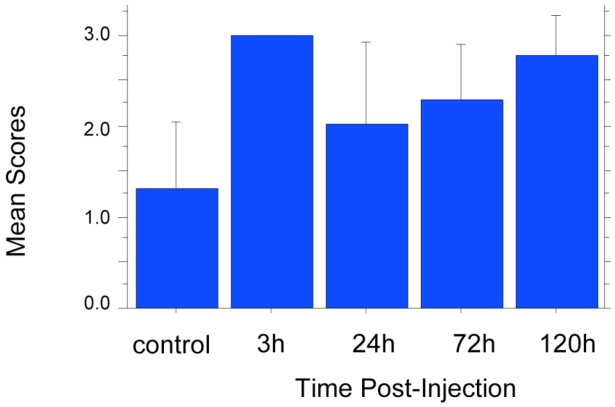
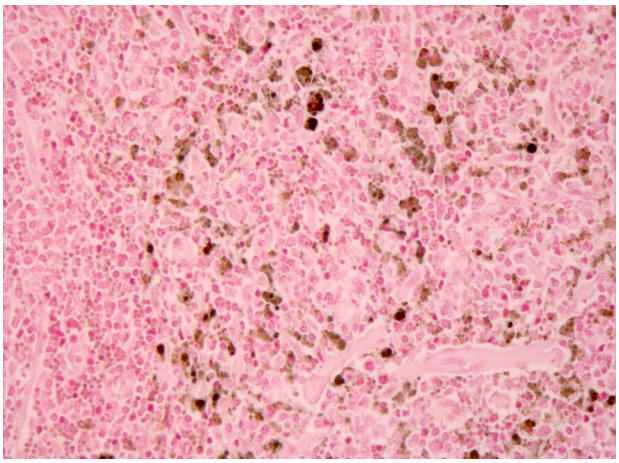


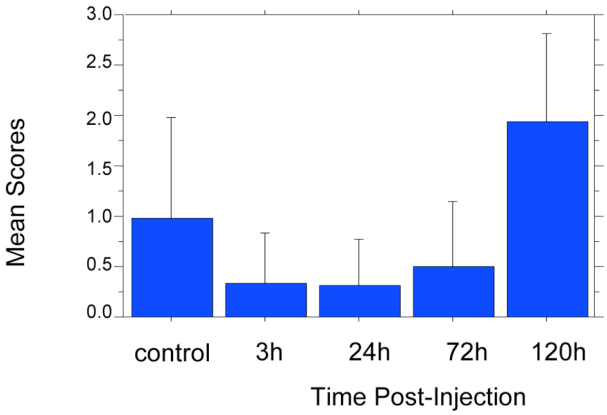
Figure 3. Distribution of detected iron ions (Fe^{2+}) in Turnbull stained lymph node tissue of controls and of animals injected with SPION.





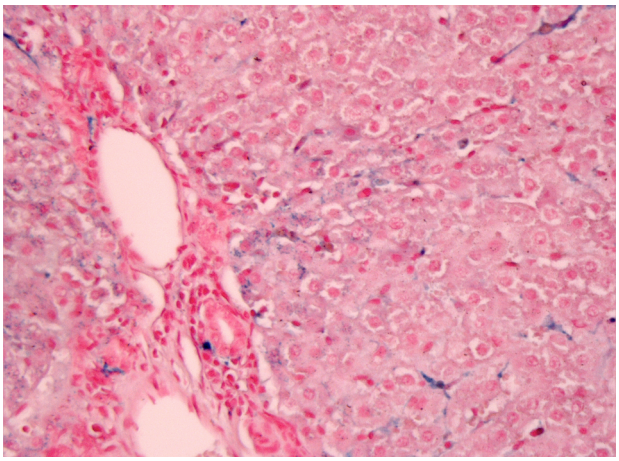
a **b**



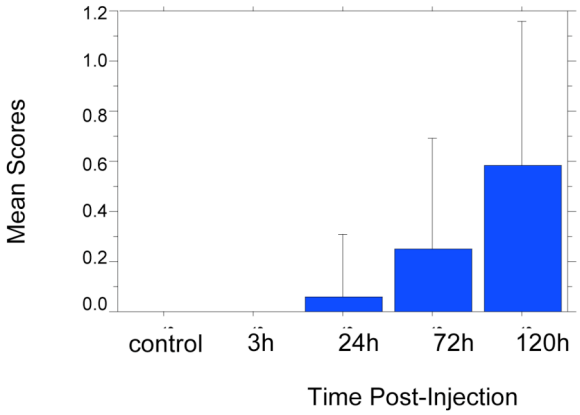


c **d**

Figure 4. Spleen tissue; *a*, blue stained macrophages 3 hours post PVA-SPION injection (P.B., 20x); *b*, distribution of detected iron ions (Fe^{3+}) in P.B. stained spleen tissue of controls and of animals injected with SPION; *c*, brown stained macrophages 3 hours post PVA-SPION injection (Turnbull, 20x); *d*, distribution of detected iron ions (Fe^{2+}) in Turnbull stained spleen tissue of controls and of animals injected with SPION.



a



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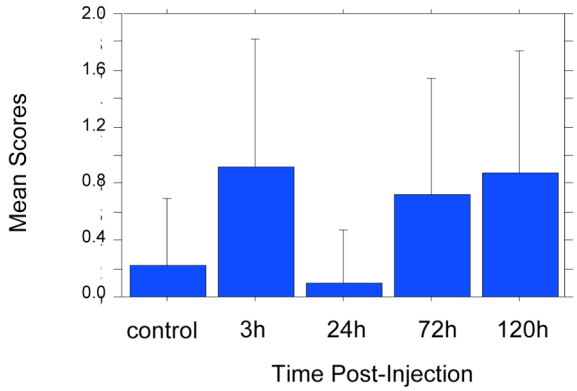

c

Figure 5. Liver tissue; *a*, blue stained Kupffer cells and periportal macrophages 120 hours post PVA-SPION injection (P.B., 20x); *b*, distribution of detected iron ions (Fe^{3+}) in P.B. stained liver tissue of controls and of animals injected with SPION; *c*, distribution of detected iron ions (Fe^{2+}) in Turnbull stained liver tissue of controls and of animals injected with SPION.

Zusammenfassung:

PVA umhüllte und fluoreszierend gefärbte (Cy 3.5) funktionalisierte Vinyl Alkohol/ Vinyl Amin Copolymer behüllte superparamagnetische Eisenoxid Nanopartikel (superparamagnetic iron oxide nanoparticles: SPION) wurden auf systemische Verteilung und Elimination nach intraartikulärer Injektion bei Schafen untersucht. Die Untersuchungen wurden 3, 24, 72 und 120 Stunden nach der Injektion mittels Lichtmikroskop, Fluoreszenzmikroskop und confokalem Mikroskop durchgeführt. Dabei konnte kein pathologischer Einfluss der SPION auf die gewonnenen Gewebeproben erkannt werden. Signifikant erhöhte Eisengehalte nach SPION-Injektion wurden in Nieren, Lymphknoten und Milz beobachtet. Dagegen wurden keine Eisenpartikel in Leber, Harnblase und Gallenblase entdeckt. Ein positives fluoreszierendes Signal konnte in keinem Organ den SPION zugeordnet werden. Unsere Ergebnisse deuten daraufhin, dass der Eisenanteil der SPION im Anschluss an eine Rückresorption im proximalen Tubulussystem der Nieren im physiologischen Eisenstoffwechsel verarbeitet werden kann, und dass die Konzentration des Cy3.5 im Körper zu niedrig war um nachgewiesen werden zu können.

Lebenslauf

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